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AlphaFold2 and STRING-Based Mapping of REJ-Mediated Interactions in PC1 Uncovers Intracellular Roles in ADPKD

Hala Salim Sonbol[1*]

[1]Department of Biochemistry, Faculty of Science, King Abdulaziz University, Kingdom of Saudi Arabia. P.O. Box No. 122522, Jeddah, 21332, Saudi Arabia

*Corresponding author: Hala Salim Sonbo (e-mail: hsonbol@kau.edu.sa).

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Abstract: Polycystin-1 (PC1), a large membrane glycoprotein encoded by PKD1, is mutated in ~85% of autosomal dominant polycystic kidney disease (ADPKD) cases. It forms a ciliary ion channel with polycystin-2. PC1's extracellular REJ domain, homologous to a sea urchin egg-sperm binding domain, has unclear intracellular roles. This study identifies its protein interactome to reveal links to ADPKD pathogenesis. The author employed a combined biochemical and computational strategy. A recombinant PET21-MBP(TEV)-REJ fusion protein (encompassing the human REJ segment) was expressed in *E. coli*, purified, and used as bait in pull-down assays with HEK293 cell lysates. Interacting proteins were identified using MALDI-TOF mass spectrometry (peptide mass fingerprinting), revealing four novel REJ-binding partners: YWHAZ (14-3-3ζ, a signaling adaptor protein), DNAH11 (dynein heavy chain 11, a ciliary motor protein), PPIA (cyclophilin A, a peptidyl-prolyl isomerase), and PCYOX1 (prenylcysteine oxidase 1 enzyme). Functional relationships among these proteins were assessed using STRING network analysis and gene ontology enrichment, identifying associations with ciliary trafficking, signal transduction, oxidative metabolism, and protein homeostasis. AlphaFold2 modelling predicted a stable multimeric complex involving REJ, PPIA, YWHAZ, and PCYOX1, with PPIA occupying a central structural role. Interface visualisation using Pymol suggested stabilising hydrogen bonding and hydrophobic interactions. Disease enrichment revealed significant links to ADPKD and related ciliopathies. These findings support a broader role for the REJ domain in intracellular signalling and protein complex formation, expanding the current understanding of PC1 function beyond its extracellular interactions and suggesting novel directions for therapeutic targeting in ADPKD.

Key Words: Polycystin-1, REJ Domain, ADPKD, Protein–Protein Interactions, AlphaFold2, STRING Database, Pull-Down Assay, Ciliary Function, Oxidative Stress, PPIA

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is a prevalent genetic disorder characterised by the progressive formation of renal cysts, ultimately leading to kidney failure. Mutations in the PKD1 gene encoding polycystin-1 (PC1) are the primary cause of ADPKD, affecting millions globally [1]. The protein products of these genes—polycystin-1, polycystin-2, and fibrocystin—are localised to the primary cilium or involved in trafficking proteins to it, underscoring their importance in ciliary signalling and development [2].

While PC1 has been predominantly studied in renal function, emerging evidence suggests that its role extends beyond kidney, impacting other organs such as the liver, pancreas, and nervous system [3]. The extracellular region of PC1 contains several domains involved in cell-cell and cell-matrix interactions, critical for signalling and structural integrity. Among these, the receptor of egg jelly (REJ) domain stands out due to its putative role in intracellular signaling. Mutations within this domain have been linked to defects in protein processing and trafficking, yet its structural and functional attributes remain poorly defined. Thus, elucidating the REJ domain's role provides a crucial step in bridging PC1's diverse organ-specific functions with ADPKD pathology [4,5].

Despite its developmental conservation and role in GPS cleavage, the intracellular relevance of the REJ domain remains under debate. The REJ domain is involved in cleavage at the G-protein-coupled proteolytic site (GPS), a prerequisite for the functional maturation of PC1 [6,7,8]. However, little is known about the intracellular binding proteins of the REJ domain, despite its biological significance.

To address this gap, we mapped the intracellular interactome of the REJ domain using pull-down assays coupled with mass spectrometry, identifying four key binding partners: YWHAZ, DNAH11, PPIA, and PCYOX1. These novel interactions broaden the functional landscape of PC1, implicating the REJ domain in diverse cellular processes including signal transduction, intracellular trafficking, oxidative stress regulation, and protein homeostasis.

YWHAZ (14-3-3 ζ/δ) is a phospho-serine/threonine-binding protein that forms a signalling scaffold regulating various cellular pathways, including apoptosis, motility, and cytoskeletal dynamics. These proteins are found throughout the cell and influence their targets' localization, stability, and molecular interactions [9]. Complementing this role in cellular regulation, DNAH11 (Dynein Heavy Chain 11) acts as a motor protein in the outer dynein arm of motile cilia, where it is essential for generating ciliary motion and plays a crucial role in mucociliary clearance within the respiratory tract [10,11]. Enhancing this network of cellular maintenance, PPIA (Peptidyl-Prolyl Cis-Trans Isomerase A), also known as cyclophilin A, assists in protein folding and the stress response. Beyond its isomerase function, it actively participates in intracellular signaling, inflammation, and apoptosis [12,13], and is notably expressed in vascular smooth muscle cells, where it contributes to reactive oxygen species (ROS)-mediated inflammatory responses [14]. Expanding this functional diversity, PCYOX1 (Prenylcysteine Oxidase 1) is a flavin-dependent enzyme that cleaves prenyl-L-cysteines to produce cysteine and reactive aldehydes. Structural data highlight hydrophobic tunnels that facilitate isoprenoid binding and membrane association [15], while functional studies have linked PCYOX1 to the regulation of platelet aggregation, thrombosis, and lipid peroxidation [16].

These interactions suggest that the REJ domain of PC1 may have extensive roles beyond kidney-related pathways. DNAH11's involvement supports a role in ciliary function, while PPIA and PCYOX1 indicate protein homeostasis and oxidative stress regulation. These observations reinforce the hypothesis that the REJ domain contributes to a broader intracellular signalling network relevant to systemic disease.

This study investigates novel intracellular mechanisms of the REJ domain by combining experimental proteomics with advanced structural bioinformatics. Modern bioinformatics tools are critical for elucidating protein interaction networks and linking them to disease mechanisms. Pathway enrichment analyses reveal the biological significance of protein interactors, while molecular docking predicts structural dynamics and binding affinities [17]. In this study, we employed in silico methods such as the STRING database for protein–protein interaction (PPI) network analysis and biological process enrichment [18,19], as well as AlphaFold2 for 3d structural prediction of the REJ–protein complex [20]. Structural interfaces were visualised and analysed using PyMOL, providing detailed insights into molecular contacts and functional domains [21]. Together, these computational approaches complement our experimental results, offering a more comprehensive understanding of the REJ domain's cellular roles.

In the context of ADPKD, integrating experimental and computational approaches provides a comprehensive framework for uncovering the intracellular functions of the REJ domain. This combined strategy not only advances understanding of PC1 dysfunction but also highlights potential therapeutic targets to mitigate its downstream effects.

METHODS

MBP-REJ Pull-Down and Mass Spectrometric Analysis

A recombinant MBP-tagged REJ domain of polycystin-1 (PC1) was generated using the PET21-MBP(TEV)-REJ construct and expressed in E. coli. The fusion protein was purified via amylose affinity chromatography and its purity verified by SDS-PAGE and Coomassie staining. For pull-down assays, HEK293 cell lysates were prepared in RIPA buffer supplemented with protease inhibitors and incubated with immobilized MBP–REJ fusion protein under optimized binding conditions designed to minimize nonspecific interactions. Following multiple high-salt and detergent washes, bound protein complexes were eluted under native conditions. Eluted fractions were first resolved by SDS-PAGE, and discrete protein bands were visualized, excised, and subjected to in-gel trypsin digestion. Peptide fragments were analyzed by MALDI-TOF mass spectrometry, and protein identities were confirmed through database searching using MASCOT and cross-validated against established proteomic datasets. This workflow ensured high specificity in identifying REJ-associated proteins while reducing background contaminants [4,5,6].

Protein Selection and Interaction Analysis

Five proteins of interest—YWHAZ, DNAH11, PPIA, PCYOX1, and polycystin-1 (PKD1)—were selected based on previous pull-down assay results. Each was queried individually using the STRING database (version 12.0) to retrieve its direct protein–protein interactors. The species was set to Homo sapiens, and only high-confidence physical or functional interactions supported by text mining were included.

Network Construction

The combined set of the five initial proteins and their identified interactors was then used as input in STRING to generate a comprehensive PPI network. STRING was set to display only interactions with "text mining" evidence (to focus on literature-curated associations), with a minimum required interaction score of 0.4 (medium confidence to maximize inclusivity). Two distinct networks were generated. The first was the Functional and Physical Association Network, which displays edges representing functional and physical protein associations (default setting). The second was the Physical Complex Network, which displays edges indicating that the connected proteins are part of a direct physical complex (for validation of direct binding partners).

Enrichment Analysis

STRING was employed to conduct enrichment analysis on the resulting network [19,22]. The analysis encompassed the following categories:

- Biological Process from Gene Ontology (GO)
- Molecular Function from Gene Ontology (GO)
- Disease-Gene Associations from the DISEASES database
- Protein Domains and Features from InterPro

Protein Complex Structure Prediction Using AlphaFold2

The three-dimensional structure of a protein complex comprising 14-3-3 zeta (YWHAZ), cyclophilin A (PPIA), the REJ domain of polycystin-1 (PKD1), and prenylcysteine oxidase (PCYOX) was predicted using the AlphaFold2_multimer_v3 model [20,23].Amino acid sequences for YWHAZ (UniProt ID: P63104), PPIA (UniProt ID: P62937, the REJ domain of PKD1 (UniProt ID: P98161, residues 2146-2833, and PCYOX (UniProt ID: Q9UHG3) were retrieved from the UniProt database [24]. The sequences were concatenated into a single FASTA file, with chain boundaries explicitly defined to represent the four distinct subunits of the complex.

AlphaFold2 employs a deep learning architecture that integrates attention-based neural networks with evolutionary information to predict protein structures accurately. For multi-chain complexes, the multimer model captures inter-chain interactions by modelling pairwise residue distances and interface contacts, informed by multiple sequence alignments (MSAS) derived from genetic databases. Structure prediction was performed using the AlphaFold Server [25], a cloud-based platform for running AlphaFold2 models. The server was configured to generate MSAS and predict the complex structure, producing atomic coordinates, per-residue confidence scores (plddt, predicted Local Distance Difference Test), and predicted aligned error (PAE) matrices to assess structural and inter-chain reliability.

Multiple models were generated to account for prediction variability, and the model with the highest average plddt score was selected for analysis. Prediction quality was evaluated by analysing plddt scores (0–100 scale, with >90 indicating high confidence) and PAE matrices to confirm the accuracy of inter-chain interfaces. The stereochemical quality of the predicted structure was assessed using MolProbity [26]. to verify geometric correctness.

Visualisation and Analysis of Inter-Chain Binding Sites

The predicted protein complex structure was visualised using Pymol Version 3.1, Schrödinger, Inc.[21]. Individual chains (YWHAZ, PPIA, REJ domain, and PCYOX) were colored distinctly to highlight the complex's quaternary architecture. Inter-chain contacts were mapped using Pymol's find pairs command to identify binding sites between chains. Residue-residue contacts were calculated within a distance cutoff of 4.0 Å between heavy atoms of different chains to define interaction interfaces. Polar interactions, including hydrogen bonds, were identified using the find_hbonds function with default criteria (distance<3.5 Å, angle > 120°). Hydrophobic contacts were inferred for non-polar residues within 4.0 Å proximity.

The identified contacts were tabulated to characterise the binding interfaces between YWHAZ, PPIA, the REJ domain, and PCYOX, focusing on residues contributing to inter-chain stability. Interaction interfaces were visually inspected in Pymol to ensure consistency with PAE matrix predictions and available biochemical data [specify if applicable, e.g., known interaction motifs or literature evidence]. Structural figures of the complex and its binding sites were generated using Pymol for inclusion in the study.

Structure prediction computations were performed via the AlphaFold Server's cloud-based infrastructure, while visualisation and contact analyses were conducted on a local computer with an Intel Core i7 and 16 GB RAM. The predicted structure and inter-chain contact analysis provided insights into the molecular interactions stabilising the YWHAZ–PPIA–REJ–PCYOX complex.

RESULTS

Interaction of MBP-REJ Fusion Protein Intracellular Proteins

The pull-down assay products were resolved on 15% SDS-PAGE, and protein–protein interactions were subsequently characterized by mass spectrometry [4]. Identified bands were matched to protein entries using the UniProt database, which enabled accurate determination of molecular identities. Four candidate interactors were identified in association with the MBP–REJ fusion protein: YWHAZ, DNAH11, PPIA, and PCYOX1. Among these, three proteins—YWHAZ, PPIA, and PCYOX1—were consistently detected as direct interactors of the REJ domain, highlighting their potential functional relevance to PC1. Table 1 includes the predicted molecular weight, isoelectric point (pI), protein name, and mass for each identified protein. It also shows the potential protein candidates suggested by Uniprot and EXPASY.

STRING-Based Interaction Network Analysis of REJ Domain-Associated Proteins

The STRING database generated comprehensive protein-protein interaction (PPI) networks based on five initial proteins—YWHAZ, DNAH11, PPIA, PCYOX1, and PKD1—and their identified interactors. Two distinct network visualisations were created using a medium confidence interaction score (0.4) with text mining evidence (Figure 1).

Functional and Physical Association Network

The first network visualisation (Figure 1) displays a complex interaction landscape comprising functional and physical protein associations. The network reveals several distinct protein clusters organised around key hub proteins. Major interaction hubs include YWHAZ, which strongly connects

Table 1: Proteins Interacting with the MBP-REJ Fusion Protein Identified by MALDI-TOF MS via Pull-Down Assay.

Proteins name	UNI port accession #	MW (kDa)	Theoretical PI
YWHAZ	1433Z__HUMAN (P63104)	≈27.8	4.73
DNAH11	DYH11_HUMAN (Q96DT5)	≈520.4	6.03
PPIA	P62937_HUMAN (P62937)	≈18	7.68
PCYOX1	PCYOX_HUMAN (Q9UHG3)	≈53.9	5.89

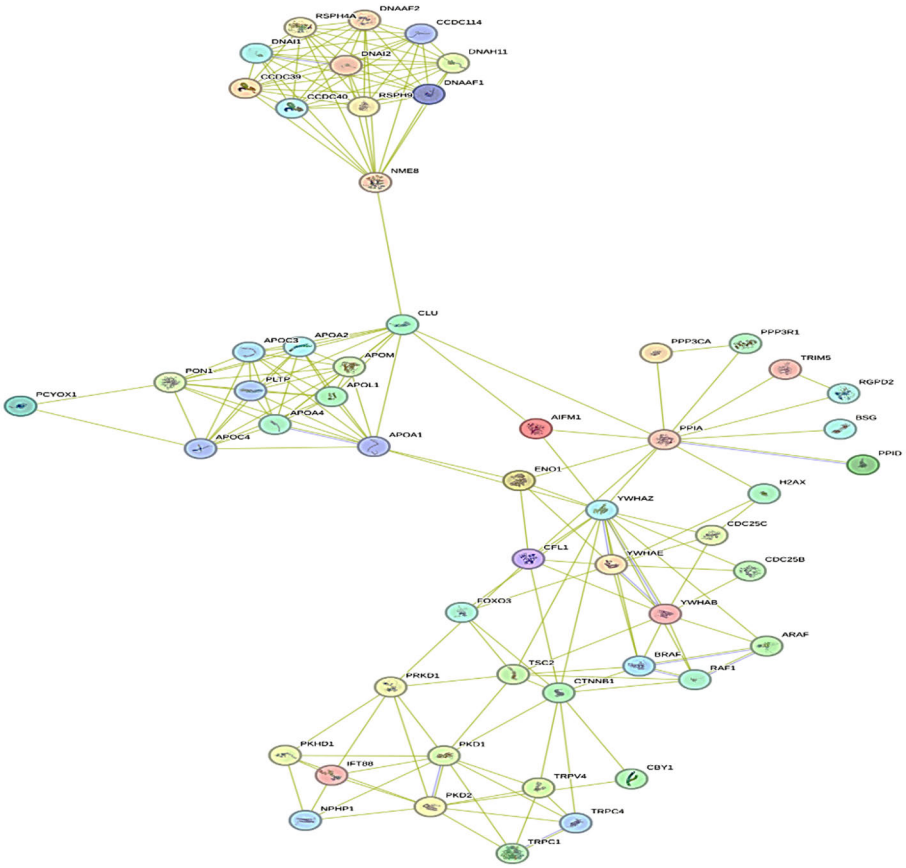

Figure 1: Based on STRING Analysis, The Functional and Physical Association Network of YWHAZ, DNAH11, PPIA, PCYOX1, PKD1, and their Interactors. This Network Includes Functional and Physical Protein Associations, Highlighting Literature-Curated Interactions with a Medium Confidence Score (0.4)

with YWHAE, YWHAB, FOXO3, and 14-3-3 family proteins, suggesting important regulatory functions. Another significant hub centres around CLU (Clusterin), interconnecting with multiple apolipoprotein family members (APOA1, APOA2, APOA4, APOC3, APOL1, APOM).

The PKD1 hub demonstrates connections with TRPC family proteins (TRPC1, TRPC4), PKD2, and PKHD1, highlighting its role in mechanosensation and calcium signalling pathways. PPIA (Peptidyl-prolyl cis-trans isomerase A) forms interactions with proteins involved in cellular stress responses, including H2AX and PPID.

Physical Complex Network

The second network visualisation (Figure 2) highlights direct physical protein complexes, revealing a reorganised topology with more defined functional modules. This network demonstrates stronger clustering patterns, particularly evident in the NME8-centered complex that includes DNAAF1, RSPH4A, DNAI1, DNAI2, DNAH11, CCDC114, and CCDC39/40— proteins predominantly associated with ciliary function and dynein arm assembly.

The apolipoprotein cluster around CLU appears more tightly connected in this representation, suggesting these proteins form actual physical complexes rather than just functional associations. Similarly, the YWHAZ/14-3-3 protein cluster shows pronounced interactions with FOXO3, CFL1, and CDC25 proteins, indicating direct physical binding relationships critical for cell cycle regulation and signal transduction.

The PKD1-centred cluster maintains strong physical interactions with TRPV4, PKD2, and PKHD1, confirming the formation of polycystin complexes essential for renal tubular development and function. Interestingly, PPIA demonstrates fewer but more specific physical interactions in this network compared to the functional association network. Both networks highlight the multifunctional nature of the initial query proteins, revealing their involvement in diverse

Table 2: Kidney/Renal Related Biological Processes

GO Term ID	Term Description	FDR	Genes
GO:0051179	Localisation	4.14E-11	TSC2, APOC3, PPP3R1, APOA1, PKD2, PKD1, YWHAE, AIFM1, DNAAF2, PPID, NPHP1, CLU, APOL1, IFT88, BSG, APOA4, APOA2, PKHD1, YWHAB, APOM, DNAAF1, PPP3CA, YWHAZ, CCDC40, RGPD2, PCYOX1, PRKD1, RAF1, TRPV4, PLTP, CCDC39, BRAF, TRPC1, CFL1, APOC4, DNAH11, CBY1, DNAI1, TRPC4, CTNNB1
GO:0043933	Protein-containing complex organisation	1.11E-10	APOC3, RSPH4A, APOA1, PKD2, PKD1, AIFM1, DNAAF2, PPID, CLU, CCDC114, APOA4, APOA2, RSPH9, APOM, DNAAF1, CCDC40, DNAI2, RAF1, TRPV4, PLTP, CCDC39, CFL1, H2AX, CBY1, DNAI1
GO:0051234	Establishment of localisation	1.11E-10	TSC2, APOC3, PPP3R1, APOA1, PKD2, PKD1, YWHAE, AIFM1, DNAAF2, PPID, CLU, APOL1, IFT88, APOA4, APOA2, PKHD1, YWHAB, APOM, DNAAF1, PPP3CA, YWHAZ, CCDC40, RGPD2, PCYOX1, PRKD1, RAF1, TRPV4, PLTP, CCDC39, BRAF, TRPC1, CFL1, APOC4, DNAH11, DNAI1, TRPC4, CTNNB1
GO:0003341	Cilium movement	2.33E-10	NME8, RSPH4A, DNAAF2, CCDC114, RSPH9, DNAAF1, CCDC40, DNAI2, PLTP, CCDC39, DNAH11, DNAI1
GO:0065003	Protein-containing complex assembly	3.93E-10	APOC3, RSPH4A, APOA1, PKD2, PKD1, AIFM1, DNAAF2, PPID, CLU, CCDC114, APOA4, APOA2, RSPH9, APOM, DNAAF1, CCDC40, DNAI2, RAF1, TRPV4, CCDC39, H2AX, CBY1, DNAI1
GO:0006810	Transport	8.90E-10	TSC2, APOC3, PPP3R1, APOA1, PKD2, PKD1, YWHAE, AIFM1, DNAAF2, PPID, CLU, APOL1, IFT88, APOA4, APOA2, YWHAB, APOM, DNAAF1, PPP3CA, YWHAZ, CCDC40, RGPD2, PCYOX1, PRKD1, RAF1, TRPV4, PLTP, CCDC39, BRAF, TRPC1, APOC4, DNAH11, DNAI1, TRPC4, CTNNB1
GO:0022607	Cellular component assembly	1.16E-09	NME8, APOC3, RSPH4A, APOA1, PKD2, PKD1, AIFM1, DNAAF2, PPID, CLU, CCDC114, IFT88, APOA4, APOA2, PKHD1, RSPH9, APOM, DNAAF1, YWHAZ, CCDC40, DNAI2, RAF1, TRPV4, CCDC39, BRAF, H2AX, CBY1, DNAI1, CTNNB1
GO:0044782	Cilium organisation	1.60E-09	NME8, RSPH4A, PKD2, DNAAF2, CCDC114, IFT88, PKHD1, RSPH9, DNAAF1, CCDC40, DNAI2, CCDC39, CBY1, DNAI1
GO:0033036	Macromolecule localisation	2.29E-09	TSC2, APOC3, PPP3R1, APOA1, PKD2, PKD1, YWHAE, AIFM1, PPID, NPHP1, CLU, APOL1, BSG, APOA4, APOA2, YWHAB, APOM, PPP3CA, YWHAZ, RGPD2, RAF1, PLTP, CCDC39, BRAF, APOC4, DNAH11, CBY1, CTNNB1
GO:0007017	Microtubule-based process	4.23E-09	NME8, RSPH4A, PKD2, DNAAF2, CCDC114, IFT88, PKHD1, RSPH9, DNAAF1, CCDC40, DNAI2, TRPV4, PLTP, CCDC39, CFL1, DNAH11, DNAI1, CTNNB1
GO:0060271	Cilium assembly	6.44E-09	NME8, RSPH4A, DNAAF2, CCDC114, IFT88, PKHD1, RSPH9, DNAAF1, CCDC40, DNAI2, CCDC39, CBY1, DNAI1
GO:0035082	Axoneme assembly	1.05E-08	RSPH4A, DNAAF2, CCDC114, RSPH9, DNAAF1, CCDC40, DNAI2, CCDC39, DNAI1
GO:0034375	High-density lipoprotein particle remodelling	2.53E-08	APOC3, APOA1, APOA4, APOA2, APOM, PLTP
GO:0007018	Microtubule-based movement	3.93E-08	NME8, RSPH4A, DNAAF2, CCDC114, IFT88, RSPH9, DNAAF1, CCDC40, DNAI2, PLTP, CCDC39, DNAH11, DNAI1
GO:0043691	Reverse cholesterol transport	3.93E-08	APOC3, APOA1, CLU, APOA4, APOA2, APOM
GO:0070286	Axonemal dynein complex assembly	3.93E-08	DNAAF2, CCDC114, DNAAF1, CCDC40, DNAI2, CCDC39, DNAI1
GO:0030030	Cell projection organisation	9.04E-08	NME8, RSPH4A, PKD2, DNAAF2, NPHP1, CCDC114, IFT88, BSG, APOA4, PKHD1, RSPH9, DNAAF1, PPP3CA, CCDC40, DNAI2, CCDC39, CBY1, DNAI1, CTNNB1
GO:0000226	Microtubule cytoskeleton organisation	1.37E-07	RSPH4A, PKD2, DNAAF2, CCDC114, PKHD1, RSPH9, DNAAF1, CCDC40, DNAI2, TRPV4, CCDC39, CFL1, DNAI1, CTNNB1
GO:0060285	Cilium-dependent cell motility	1.37E-07	NME8, RSPH4A, DNAAF2, RSPH9, CCDC40, PLTP, CCDC39, DNAH11, DNAI1
GO:0016043	Cellular component organisation	3.00E-07	NME8, APOC3, RSPH4A, APOA1, PKD2, CDC25B, PKD1, AIFM1, DNAAF2, PPID, NPHP1, CLU, CCDC114, IFT88, BSG, APOA4, APOA2, PKHD1, RSPH9, APOM, DNAAF1, PPP3CA, YWHAZ, CCDC40, PRKD1, DNAI2, RAF1, TRPV4, PLTP, CCDC39, BRAF, PPIA, CFL1, H2AX, CBY1, DNAI1, CTNNB1
GO:0120036	Plasma membrane-bound cell projection organisation	3.00E-07	NME8, RSPH4A, PKD2, DNAAF2, CCDC114, IFT88, BSG, APOA4, PKHD1, RSPH9, DNAAF1, PPP3CA, CCDC40, DNAI2, CCDC39, CBY1, DNAI1, CTNNB1
GO:0007275	Multicellular organism development	9.23E-07	TSC2, PPP3R1, APOA1, PKD2, PKD1, YWHAE, AIFM1, DNAAF2, NPHP1, CLU, IFT88, BSG, FOXO3, APOA4, PKHD1, DNAAF1, PPP3CA, YWHAZ, CCDC40, PRKD1, DNAI2, RAF1, TRPV4, CCDC39, BRAF, PPIA, H2AX, DNAH11, CBY1, DNAI1, TRPC4, CTNNB1
GO:0060294	Cilium movement is involved in cell motility	1.19E-06	NME8, RSPH4A, RSPH9, CCDC40, PLTP, CCDC39, DNAH11, DNAI1
GO:0003351	Epithelial cilium movement is involved in extracellular fluid movement	1.45E-06	DNAAF2, DNAAF1, CCDC40, CCDC39, DNAH11, DNAI1
GO:0071702	Organic substance transport	1.45E-06	TSC2, APOC3, PPP3R1, APOA1, PKD1, YWHAE, AIFM1, PPID, CLU, APOL1, APOA4, APOA2, YWHAB, APOM, PPP3CA, YWHAZ, RGPD2, RAF1, TRPV4, PLTP, APOC4, TRPC4

Table 2: Continue

GO:0007368	Determination of left/right symmetry	1.60E-06	PKD2, DNAAF2, DNAAF1, CCDC40, DNAI2, CCDC39, DNAH11, DNAI1
GO:0015918	Sterol transport	1.84E-06	APOC3, APOA1, CLU, APOA4, APOA2, APOM, PLTP
GO:0032501	Multicellular organismal process	1.84E-06	NME8, TSC2, APOC3, PPP3R1, APOA1, PKD2, CDC25B, PKD1, YWHAE, AIFM1, DNAAF2, PPID, NPHP1, CLU, CCDC114, IFT88, BSG, FOXO3, APOA4, APOA2, PKHD1, RSPH9, APOM, DNAAF1, PPP3CA, YWHAZ, CCDC40, PRKD1, DNAI2, RAF1, TRPV4, PLTP, CCDC39, BRAF, PPIA, CFL1, H2AX, CBY1, DNAI1, TRPC4, CTNNB1

Table 3: Kidney/Renal Related Molecular Function

GO Term ID	Term Description	FDR	Genes
GO:0005262	Calcium Channel Activity	0.0045	PKD2, PKD1, TRPV4, TRPC1, TRPC4
GO:0044325	Transmembrane Transporter Binding	0.00098	PKD2, PKD1, YWHAE, YWHAZ, TRPC1, CTNNB1
GO:0005319	Lipid Transporter Activity	0.002	APOA1, APOA4, APOA2, APOM, PLTP, APOC4
GO:0015485	Cholesterol Binding	0.0045	APOC3, APOA1, APOA4, APOA2
GO:0019899	Enzyme Binding	0.00027	TSC2, PKD2, PKD1, CTNNB1
GO:0042803	Protein Homodimerization Activity	0.0213	PKD2, APOA1, APOA4, APOA2

Table 4. Kidney/Renal Related Diseases

DOID	Term Description	FDR	Genes
DOID:0080322	Polycystic Kidney Disease	2.85e-7	PKD2, PKD1, IFT88, PKHD1, PRKD1
DOID:557	Kidney Disease	2.33E-06	TSC2, PKD2, PKD1, NPHP1, APOL1, IFT88, APOA4, PKHD1, PRKD1, CTNNB1
DOID:0110861	Autosomal Recessive Polycystic Kidney Disease	2.86E-06	PKD2, PKD1, PKHD1, PRKD1
DOID:898	Autosomal Dominant Polycystic Kidney Disease	4.32E-06	PKD2, PKD1, PKHD1, PRKD1
DOID:0110858	Polycystic Kidney Disease Type 1	0.0092	PKD2, PKD1
DOID:0110859	Polycystic Kidney Disease Type 2	0.0092	PKD2, PKD1

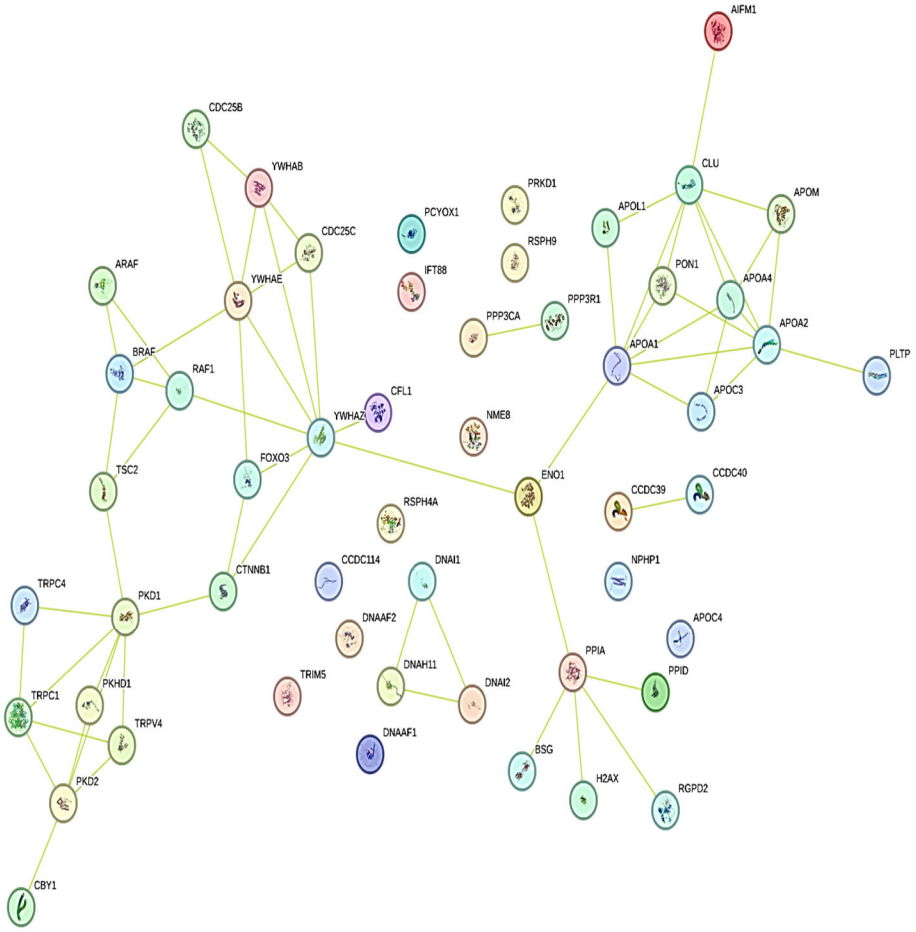

Figure 2: Based on STRING *A*nalysis, the *Physical Complex Network* of YWHAZ, DNAH11, PPIA, PCYOX1, PKD1, and their *I*nteractors. This *Network Focuses on Direct Physical Complexes Among the Proteins to Validate Binding Partners with a Medium Confidence Score* (0.4)

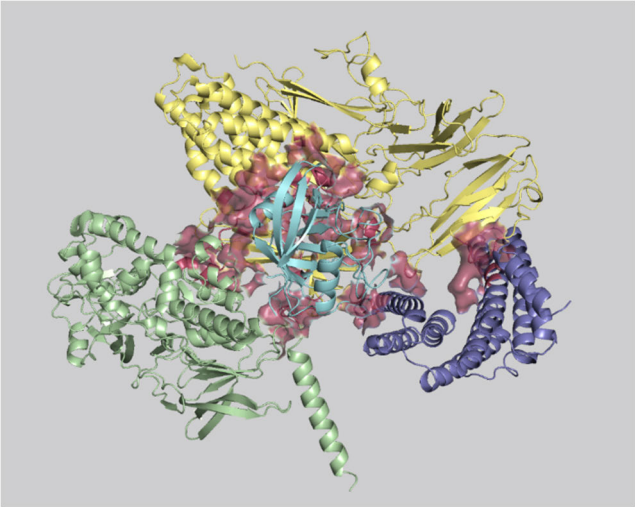

Figure 3: Predicted YWHAZ–PPIA–REJ–PCYOX Protein Complex Structure. The Structure was Predicted using AlphaFold2 via the AlphaFold Server and Visualised in Pymol. Chains are Colored as follows: REJ Domain of Polycystin-1 (Yellow), PCYOX (Green), PPIA (Cyan), and YWHAZ (Blue). Red regions Highlight Inter-Chain Interaction Interfaces (Residues within 4.0 Å). PPIA Acts as a Central Hub, Mediating Interactions with All Three Other Chains, With the Rej Domain Showing Flexible Loops and YWHAZ Providing a Helical Scaffold

cellular processes, including signal transduction, lipid metabolism, ciliary function, and cellular stress responses through extensive protein interaction networks.

Biological Process Enrichment Analysis

The biological process enrichment analysis reveals significant connections to kidney and renal-related functions (Table 2). Several enriched GO terms point to the importance of cilia and transport processes in kidney biology and processes related to localisation, cellular component organisation, and kidney development.

While Kidney development (GO:0003059) and Kidney morphogenesis (GO:0072000) were not significantly enriched in this particular dataset based on the specified parameters ($p<0.05$ and text mining evidence), Multicellular organism development (GO:0007275) is a highly significant term that encompasses kidney development. The presence of genes like PKD1, PKD2, NPHP1, IFT88, and PKHD1 within this category underscores the importance of these genes in the proper development of the kidney. Disruptions in these genes can lead to congenital kidney malformations and diseases.

Several of the top enriched GO terms are related to localisation (GO:0051179, GO:0051234, GO:0033036) and cellular component organisation (GO:0043933, GO:0065003, GO:0022607, GO:0016043, GO:0120036). These broad terms indicate a coordinated effort in the cell to organise its components and traffic proteins to specific locations. This is essential for proper kidney function, as different proteins must be localised to specific compartments within kidney cells to carry out their roles in filtration, transport, and signalling. These processes are critical during kidney development as cells differentiate and organise into functional units.

A large proportion of the enriched GO terms are related to cilia, including "cilium movement" (GO:0003341), "cilium organisation" (GO:0044782), "cilium assembly" (GO:0060271), "axoneme assembly" (GO:0035082), "microtubule-based movement"(GO:0007018), "axonemal dynein complex assembly"(GO:0070286), "cilium-dependent cell motility" (GO:0060285), "cilium movement involved in cell motility"(GO:0060294), and "epithelial cilium movement involved in extracellular fluid movement"(GO:0003351). Cilia are hair-like structures on the surface of many kidney cells and play a crucial role in sensing fluid flow, mediating signalling pathways, and establishing left-right asymmetry during development. Disruptions in ciliary function can lead to various kidney diseases, including polycystic kidney disease (PKD) and nephronophthisis. Cilia also play a vital role in kidney development, guiding cell differentiation and tissue organisation.

Several GO terms are related to transport, including "transport" (GO:0006810), "organic substance transport" (GO:0071702), "sterol transport" (GO:0015918), "high-density lipoprotein particle remodelling" (GO:0034375), and "reverse cholesterol transport" (GO:0043691). The kidney is responsible for filtering blood and regulating the excretion of various substances, so disruptions in transport processes can lead to kidney disease. The presence of "sterol transport" and "cholesterol transport" terms suggests a role for lipid metabolism in kidney function. Lipids play a role in kidney diseases such as focal segmental glomerulosclerosis (FSGS). Proper transport is also necessary for kidney development, as nutrients and signalling molecules must be transported to developing kidney cells. Finally, "determination of left/right symmetry" (GO:0007368) is relevant because defects in this process, which relies on cilia, can lead to kidney malformations.

Molecular Function Enrichment Analysis

The enrichment analysis of molecular functions revealed several key processes associated with proteins implicated in kidney and renal biology. Table 3 shows the records of the most statistically significant findings.

Notable Overlaps with Kidney Disease Mechanisms

APOL1 (observed in "Transporter Activity"), Variants in this gene are strongly associated with chronic kidney disease in populations of African ancestry [27]. Additionally, IFT88 (observed in "Protein Binding") is critical for cilia function in renal tubular cells, mutations lead to nephronophthisis [28]. TRPV4/TRPC Channels: Modulate calcium influx in podocytes and tubular cells, influencing renal injury responses [29].

Statistical Highlights

Most Enriched Term: High-density lipoprotein particle receptor binding (GO:0070653) with enrichment strength =

2.56 (FDR = 4.7×10^(-4)). Lowest FDR: Enzyme binding (GO:0019899) at 2.7×10^(-4).

In conclusion, the recurrence of polycystins (PKD1/PKD2) and apolipoproteins highlights their dual roles in renal physiology and disease.

Diseases such as Polycystic Kidney Disease and its subtypes (Autosomal Dominant and Autosomal Recessive) exhibit high enrichment strength (>2), indicating strong associations between the observed genes and these conditions. The false discovery rates are exceptionally low, suggesting high statistical significance for the associations. The matching proteins across these diseases frequently include key genes such as PKD2 and PKD1, which are well-known for their roles in kidney function and pathology. This enrichment analysis highlights significant associations between key genes and kidney-related diseases, particularly polycystic kidney disease and its variants. The identified proteins (PKD2, PKD1, IFT88, PKHD1, PRKD1) play critical roles in renal health and disease mechanisms, providing valuable insights for further research into kidney disorders and potential therapeutic targets.

Protein Complex Structure Prediction and Visualisation

The predicted structure of the protein complex comprising 14-3-3 zeta (YWHAZ), cyclophilin A (PPIA), the REJ domain of polycystin-1 (PKD1), and prenylcysteine oxidase (PCYOX) was obtained using AlphaFold2 and visualised in Pymol (Figure 3). The complex revealed a compact quaternary assembly with distinct structural features for each chain: the REJ domain (yellow), PCYOX (green), PPIA (cyan), and YWHAZ (blue). Inter-chain interaction interfaces, defined as residues within 4.0 Å of another chain, were highlighted in red to map binding sites.

Enrichment Analysis of Diseases

The enrichment analysis identified several diseases associated with kidney and renal issues. These diseases were filtered based on their term descriptions containing "kidney" or "renal." Table 4 details the findings.

PPIA emerged as a central hub within the complex, mediating interactions with all three other chains. Structurally, PPIA exhibited a compact beta-barrel fold, consistent with its known architecture as a peptidyl-prolyl isomerase, with interaction sites distributed across its surface loops and beta-sheet edges. The REJ domain displayed a mix of alpha helices and extended, flexible loops, indicating its role as a protein-protein interaction module in Polycystin-1. These loops were frequently involved in contacts with PPIA and PCYOX, suggesting conformational adaptability in binding. PCYOX adopted a predominantly helical structure, with a prominent alpha-helical bundle that contributed to its interaction interfaces, particularly with PPIA. YWHAZ, a member of the 14-3-3 protein family, formed a helical bundle that provided a scaffold-like role, primarily interacting with PPIA and, to a lesser extent, PCYOX.

Inter-chain contacts, highlighted in red, were concentrated at the central interface where all four chains converged, underscoring PPIA's pivotal role in complex assembly. The PPIA–REJ interface involved the REJ domain's flexible loops wrapping around PPIA's beta-barrel, with hydrogen bonds and hydrophobic contacts likely stabilising the interaction. Similarly, the PPIA–PCYOX interface featured PCYOX's alpha helices docking onto PPIA's surface, with hydrophobic interactions inferred from the proximity of non-polar residues. The PPIA–YWHAZ interface showed YWHAZ's helical groove engaging PPIA, consistent with 14-3-3 proteins' typical binding mode, potentially involving specific residues such as phosphorylated sites on PPIA. Secondary interfaces, such as those between PCYOX–REJ and PCYOX–YWHAZ, were less extensive but contributed to overall stability, with smaller red patches indicating minor contact points.

The REJ domain's disordered loops suggested regions of lower prediction confidence, as expected for flexible segments in AlphaFold2 models, but the core interaction interfaces displayed well-defined secondary structures, supporting the reliability of the predicted binding sites. The compact arrangement of the complex, with PPIA at the centre, indicated a cooperative assembly mechanism, where PPIA likely facilitates the recruitment and stabilisation of the REJ domain, PCYOX, and YWHAZ into a functional unit.

DISCUSSION

The present study provides novel insights into the intracellular interactions of the REJ domain of PC1, a membrane protein central to the pathogenesis of ADPKD. Using pull-down assays combined with mass spectrometry, we identified four previously unreported REJ-interacting proteins: YWHAZ (14-3-3ζ), DNAH11, PPIA, and PCYOX1. Functional annotation through STRING network analysis suggested that these proteins participate in critical pathways such as signal transduction, ciliary trafficking, oxidative metabolism, and protein folding. To complement these findings, AlphaFold2-based structural modelling predicted a stable multiprotein complex involving the REJ domain, with PPIA occupying a central stabilizing position. Together, these results highlight the broader cellular role of the REJ domain beyond extracellular interactions and suggest new avenues for exploring PC1 dysfunction in ADPKD.

REJ–YWHAZ Interaction: Linking Signal Transduction, Calcium Homeostasis, and mTOR Dysregulation in ADPKD

The interaction between the REJ domain and YWHAZ is notably important. YWHAZ is part of the 14-3-3 protein family, which has vital functions in signal transduction, cell cycle regulation, and apoptosis [30]. Research in biochemistry and molecular biology indicates that more than 50 signalling molecules can be affected by the 14-3-3 protein [30]. Through its interactions with effectors, 14-3-3 has become acknowledged as essential for regulating various cell signalling pathways. This includes critical processes such as activating protein kinases, managing the cell cycle, supporting neural development, and even influencing the

pathogenesis of bacteria and viruses [31]. In kidney tissues, the 14-3-3 proteins affect ion channel function and control calcium homeostasis [32,33,34]. Both of these processes are impaired in ADPKD. Our results indicate that the REJ-YWHAZ interaction may influence calcium signalling or ciliary function, aligning with the established role of PC1 as a mechanosensor on renal primary cilia [35]. Additionally, YWHAZ has been identified as a participant in mTOR pathway regulation [36], which is frequently hyperactivated in polycystic kidney disease, suggesting a potential mechanistic connection between REJ interaction and cyst formation.

REJ–DNAH11 Interaction: Implications for Ciliary Function and Cystogenesis

A significant interaction was recognised involving DNAH11, a dynein heavy chain crucial for ciliary movement and intracellular transport. Mutations in DNAH11 are associated with primary ciliary dyskinesia [37], and issues with ciliary motility are strongly linked to the pathophysiology of cystic kidney diseases. In the context of ADPKD, PC1 and PC2 come together at the cilium to regulate fluid flow-induced signalling [38]. Their relationship with DNAH11 might influence the backwards transport or positioning of the PC1 complex, thereby affecting signal transduction and the abnormal behaviour of tubular epithelial cells, ultimately leading to cyst formation and disease progression.

Classical links associate DNAH11 mutations with primary ciliary dyskinesia (PCD), a disorder characterised by immotile cilia leading to chronic respiratory infections and situs inversus [37]. Significantly, individuals with PCD caused by DNAH11 mutations show overlapping renal phenotypes, suggesting a possible common pathophysiological mechanism related to impaired ciliary transport or assembly [39]. The interaction between DNAH11 and the REJ domain of PC1 may indicate a role in either retrograde trafficking or the positional regulation of the PC1/PC2 complex within the cilium. Dynein-mediated retrograde transport is crucial for intraflagellar transport (IFT), a bidirectional process essential for maintaining the composition and signalling of cilia [40]. Should DNAH11 influence PC1 positioning or transport within the cilium, its dysfunction or misinteraction could disrupt flow-sensitive mechanotransduction, potentially encouraging cyst formation. Moreover, ciliary dyneins line up with microtubule anchoring complexes and IFT particle assemblies [41], which can influence the structural domains of cargo proteins such as PC1. Although traditionally associated with extracellular adhesion and signalling, the REJ domain may have scaffold-like properties that support the recruitment of dynein components such as DNAH11.

The identification of the REJ–DNAH11 interaction in the pulldown assay suggests that PC1 might indirectly regulate dynein function or ciliary structure, in addition to its established receptor-like functions. If this interaction is disrupted, it may result in abnormal ciliary length, protein buildup, or signal misrouting, all of which are noted in ADPKD pathology [42]. This mechanistic insight supports the idea that ciliary transport and PC1–dynein coordination may represent an underexplored avenue for therapeutic targeting, especially in patients with mild dynein defects or trafficking delays rather than complete gene loss.

REJ–PPIA Interaction: Implications for Protein Folding, ER Stress, and Therapeutic Targeting in ADPKD

PPIA is known for its role in facilitating the proper folding of transmembrane proteins and is involved in redox regulation [13]. Given that PC1 is a large and complex protein requiring intricate folding for optimal membrane trafficking, PPIA may contribute to the maturation and stability of PC1 within the endoplasmic reticulum (ER). The misfolding of PC1 has been associated with the onset of ADPKD[43], underscoring this interaction's importance.The relationship between the REJ domain of PC1 and PPIA suggests a significant role in the folding, stabilisation, and trafficking of PC1, which is critical for maintaining the proper function of renal epithelial cells. PPIA is a well-characterised cis-trans isomerase that facilitates the isomerisation of proline residues in polypeptide chains, a crucial step in protein folding. This process is particularly significant for the maturation of larger, membrane-spanning proteins, including ion channels and receptors [44]. Moreover, PPIA must be located in both the cytoplasm and the ER, ensuring protein quality and protecting against ER stress [45]. PC1 is a large, multi-pass membrane protein with a complex biogenesis process that necessitates post-translational modifications, correct folding, and transport through the secretory pathway. The misfolding or retention of PC1 in the ER is directly linked to the development of ADPKD by diminishing the levels of functional protein at the plasma membrane and primary cilium [46].

Chaperone-mediated folding, such as PPIA's support, is vital in preventing diseases. Additionally, PPIA is known to be involved in redox-sensitive folding mechanisms. It features cysteine residues that can undergo oxidation during oxidative stress, which may change its enzymatic activity [47]. Since ADPKD is linked to oxidative stress and ER stress [48], it's quite likely that PPIA has a dual role: helping stabilise PC1 when conditions are normal and providing support against folding issues when stress occurs. The REJ domain might function as a docking or interaction site, enhancing this regulation. From a therapeutic perspective, targeting this interaction opens up exciting new opportunities. We could consider pharmacologic chaperones or cyclophilin modulators to encourage proper PC1 folding and increase its expression at the cell surface in cystic disease models. Moreover, by modulating oxidative stress, we might also indirectly affect PPIA activity, ultimately influencing PC1 homeostasis.

REJ–PCYOX1 Interaction: Linking Oxidative Stress, Lipid Metabolism, and Therapeutic Opportunities in ADPKD

Identifying PCYOX1 as a binding partner of the REJ domain in PC1 reveals a potential new connection between REJ-mediated signalling and the regulation of cellular oxidative

stress. PCYOX1 is a flavin adenine dinucleotide (FAD)-dependent oxidase that converts prenylcysteine into cysteine, aldehyde, and hydrogen peroxide (H_2O_2), thereby influencing the levels of ROS in cells [49,50]. This enzymatic function suggests that PCYOX1 may serve as a modulator of oxidative stress by being a source of ROS. In autosomal dominant polycystic kidney disease (ADPKD), oxidative stress is increasingly recognised as a driver of renal cyst formation, inflammation, and fibrosis [48]. Research indicates that higher levels of ROS lead to abnormal cell growth and apoptosis in the epithelial cells lining the cysts. In PKD models, mitochondrial dysfunction and NADPH oxidase-derived ROS have been associated with cyst expansion and interstitial damage [51]. We've discovered that PCYOX1 acts as a REJ-interacting protein, which opens up an intriguing possibility for an intracellular regulatory mechanism. The REJ domain of PC1 might support or adjust the activity of enzymes that manage redox homeostasis. This relationship could significantly affect the production of ROS in specific subcellular areas, like the ER or peroxisomes, which are linked to oxidative damage associated with PKD [52].

Additionally, lipid metabolism, which is closely linked to PCYOX1 activity, is beginning to show its significance in renal epithelial cell signalling. The accumulation of lipids and the mismanagement of lipid oxidation contribute to cyst development by impacting energy metabolism and signalling pathways such as AMPK and mTOR [16]. This suggests that the interaction between REJ and PCYOX1 might also influence how cystic cells reprogram their metabolism. From a treatment perspective, this interaction highlights promising areas for exploration. Reducing oxidative stress and decelerating cyst progression may be possible by inhibiting the ROS production driven by PCYOX1 or modifying its location and activity through mechanisms associated with REJ. Furthermore, investigating pharmacological antioxidants or inhibitors of prenylation pathways would be advantageous in assessing their effectiveness in PC1-deficient models. To better understand how REJ might influence PCYOX1's enzymatic activity or support redox-related processes, we must conduct further mechanistic studies, including co-immunoprecipitation, redox-sensitive fluorescent probes, and CRISPR-based knockouts.

STRING analysis revealed that the identified proteins are significantly enriched in biological processes such as protein folding, intracellular trafficking, and cytoskeletal organisation—pathways vital for PC1 function and maintaining kidney epithelial cell integrity. Importantly, several proteins within the REJ-associated interaction network were linked to epithelial morphogenesis, suggesting that REJ-mediated interactions may play a broader role in shaping renal tubule architecture.

In addition, structural predictions using AlphaFold2 support the possibility of direct physical interactions between the REJ domain and the identified intracellular proteins. For example, the modelled binding orientations between REJ and YWHAZ or PPIA suggest reasonable contact sites that could contribute to PC1 stabilisation or modulate its intracellular signalling. While these structural predictions require experimental validation, they provide a valuable starting point for mutagenesis or co-crystallisation studies to confirm these interactions at the atomic level.

The identification of YWHAZ, DNAH11, PPIA, and PCYOX1 as intracellular binding partners of the REJ domain in polycystin-1 underscores a complex network by which PC1 may manage various cellular functions essential for kidney epithelial homeostasis. YWHAZ indicates a potential role in uniting calcium and mTOR signalling pathways, while DNAH11 suggests the REJ domain's participation in the positioning of cilia and intraflagellar transport dynamics. The interaction with PPIA hints at a regulatory function in the redox-sensitive folding and transport of PC1, possibly protecting it against ER stress, whereas PCYOX1 implies that the REJ domain is involved in managing oxidative stress and lipid metabolism. These associations establish the REJ domain as a pivotal intracellular scaffold stabilising PC1's function while connecting it with broader cellular stress responses and metabolic signals. This integrative approach broadens our understanding of PC1's functions beyond its ciliary location. It offers new perspectives on how disruptions within its intracellular dynamics may lead to the onset and advancement of ADPKD. Despite its novel findings, this study has one limitation i.e the AlphaFold2-based models, although informative, are computational predictions and should be interpreted as speculative until completely experimentally validated. Future investigations focusing on these REJ-protein interactions could uncover new therapeutic avenues aimed at restoring PC1 balance and reducing cyst development.

CONCLUSIONS

This study identifies YWHAZ, DNAH11, PPIA, and PCYOX1 as novel intracellular interactors of the REJ domain of PC1, linking it to key processes including signal transduction, ciliary transport, protein folding, and oxidative stress regulation. These findings expand the functional scope of the REJ domain beyond extracellular interactions, positioning it as an essential regulator of PC1's intracellular roles. Through integrative bioinformatics—STRING network analysis, Gene Ontology enrichment, and AlphaFold2-based structural modelling—supported by PyMOL visualisation, we provide both functional and structural evidence for these interactions, with PPIA highlighted as a central stabilizing partner. Collectively, this work establishes the REJ domain as a pivotal hub in PC1's protein interactome, advancing our understanding of ADPKD pathogenesis and opening promising avenues for therapeutic intervention.

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Conflict of Interest

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